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ANSWER 112 OF 129 MEDLINE
AN 80197699 MEDLINE
DN 80197699
TI Hemotherapy and ***antibacterial*** defense mechanisms.
AU Alexander J W
SO BIBLIOTHECA HAEMATOLOGICA, (1980) (46) 26-36. Ref: 18
Journal code: 9SW. ISSN: 0067-7957.
CY Switzerland
DT Journal; Article; (JOURNAL ARTICLE)
General Review; (REVIEW)
LA English
FS Priority Journals
EM 198009

L3 ANSWER 121 OF 129 MEDLINE
AN 71291866 MEDLINE
DN 71291866
TI Structure-activity relationships in membrane-perturbing agents. Hemolytic, narcotic, and ***antibacterial*** compounds.
AU Hansch C; Glave W R
SO MOLECULAR PHARMACOLOGY, (1971 May) 7 (3) 337-54.
Journal code: NGR. ISSN: 0026-895X.
CY United States
DT Journal; Article; (JOURNAL ARTICLE)
LA English
FS Priority Journals
EM 197112

8 ANSWER 75 OF 84 MEDLINE
AN 67127274 MEDLINE
DN 67127274
TI The loss of certain cellular components from human ***erythrocytes*** during hypotonic hemolysis in the presence of ***dextran*** .
AU Hjelm M; Ostling S G; Persson A E
SO ACTA PHYSIOLOGICA SCANDINAVICA, (1966 May) 67 (1) 43-9.
Journal code: 1U4. ISSN: 0001-6772.
CY Sweden
DT Journal; Article; (JOURNAL ARTICLE)
LA English
FS Priority Journals
EM 196707

VOLUME TIGHTLY BOUND

Acta physiol. scand. 1966. 67. 43—49

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The Loss of Certain Cellular Components from Human Erythrocytes during Hypotonic Hemolysis in the Presence of Dextran

By

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Abstract

HJELM, M., S. G. ÖSTLING and A. E. G. PERSSON. *The loss of certain cellular components from human erythrocytes during hypotonic hemolysis in the presence of dextran.* Acta physiol. scand. 1966. *67.* 43—49.

The escape of certain cellular components (catalase, glucose-6-phosphate dehydrogenase, hexokinase, lactate dehydrogenase, adenosine-triphosphate, glutathione) was studied in human red cells suspended in a hemolytic hypotonic salt solution containing dextran. There was in inverse relationship between size of a component and the relative amount of it escaping from the cell.

Marsden, Zade-Oppen and Johansson (1957) reported that human erythrocytes suspended in a hypotonic solution containing dextran did not release as much hemoglobin (Hb) as cells at the same tonicity without dextran. It was found that the dry masses of the cells were distributed unimodally around a new lower mean value after hemolysis. (Since the stroma and components, other than Hb, constitute only about 5 per cent of the unhemolysed red cell dry mass, the latter can be used as a fair approximation of the Hb content, provided this is not too low.) These observations were interpreted as indicating that partial liberation of Hb from all, or nearly all, the red cells occurred under such conditions. A close relationship was found between the Hb escaping and the dextran concentration in a hypotonic solution which would cause complete hemolysis in the absence of dextran (Zade-Oppen 1960 a). Thus it was possible to predict the mean loss of Hb from the erythrocyte population occurring with a particular dextran concentration in the hypotonic system.

It was also found that the cells took up dextran under these conditions (Marsden and Östling 1959). Analysis of the molecular weight distribution of the dextran fixed in the red cells showed a relative overweight of low molecular dextran in the cells. There appeared to be an upper limit of permeation at a molecular weight of

about 300,000 to 400,000, though this figure must be regarded as somewhat uncertain. It is perhaps interesting to note that Gerhardt and Black (1961) found a similar upper exclusion limit in the walls of bacterial spores.

In the present investigation the hypotonic dextran system has been utilized for studying the disappearance of molecules, other than Hb, from human erythrocytes. Catalase, glucose-6-phosphate dehydrogenase, hexokinase and lactate dehydrogenase were chosen as high molecular weight substances while adenosine-triphosphate and glutathione served as low molecular weight indicators.

Materials and Methods

Venous blood samples were collected in heparinized tubes from healthy blood donors at the Blood Transfusion Service (By kind courtesy of Dr C. F. Högman), University Hospital, Uppsala. Phosphoglycerate kinase, phosphoglyceraldehyde dehydrogenase, ATP, NADP and NADH were obtained from Boehringer & Soehne, Mannheim, West Germany. Methylglyoxal was obtained as a crude preparation from Kemikalieaktiebolaget KEBO AB, Stockholm, Sweden, and was purified by steam distillation. Dextran (Dextran 250®, $\overline{M}_w = 250{,}000$, $\overline{M}_n = 123{,}000$) was a generous gift from AB Pharmacia, Uppsala, Sweden. All other chemicals were of reagent grade.

General procedure

A measured volume of washed human erythrocytes was suspended in a relatively large volume of a hypotonic buffer solution containing varying concentrations of dextran which regulated the amounts of Hb which were released in hypotonic hemolysis. The tonicity was restored with a slightly hypertonic buffer solution. The Hb liberation was calculated as the percentage of the Hb found in the clear supernatant after the cells had subsequently been spun down in relation to the total Hb content of the hemolysate. The hemolysed cells were washed in the isotonic buffer solution and finally suspended in a small volume. This cell suspension was used for the analyses, described below.

Partial hemoglobin liberation from erythrocytes

All procedures were done at + 4 °C. The heparinized whole blood sample was centrifuged at 100 × g for 5 min (Nikkilä 1962). The plasma and leucocytes were removed. The erythrocytes were suspended in an isotonic (289 mOsm, see Hendry, 1961) buffer solution, pH 7.4, of the following composition: NaCl 116 mM, KCl 4 mM, $MgCl_2$ 5.5 mM, NaH_2PO_4 and Na_2HPO_4 20 mM (= 43 mOsm) (Hendry 1961) and glucose 10 mM. The osmolarity of the solutions was checked by measuring the freezing point depression with an osmometer (Advanced Instruments, model 64-31). After centrifugation at 1500 × g for 10 min the supernatant was discarded and one part of the sedmented erythrocytes (hematocrit about 90 per cent) was mixed with an equal part of the washing solution. One volume of the resulting suspension, equilibrated with atmospheric air, was rapidly mixed with 19 volumes of a hypotonic solution of the following composition:

4—10 per cent (w/v) dextran (measured polarimetrically) in a hypotonic (65 mOsm without dextran) buffered saline, pH 7.4, consisting of NaCl 5 mM, KCl 5 mM, NaH_2PO_4 and Na_2HPO_4 20 mM. This constituted the hemolysing system.

The total Hb concentration in this system (partially hemolysed red cells + liberated Hb) was determined as methemoglobincyanide by the method of Zade-Oppen (1960 b) after complete hemolysis of a small sample in destilled water, diluted 1 : 41.

The suspension was adjusted to isotonicity after 30 to 60 min by adding 180 volumes of a hypertonic (315 mOsm) buffered saline, pH 7.4 of the following composition: NaCl 130 mM, KCl 4 mM, $MgCl_2$ 5.5 mM, NaH_2PO_4 and Na_2HPO_4 20 mM and glucose 10 mM. The cells were spun down at 700 × g in 500 ml glass bottles or at 1500 × g in 100 ml glass test tubes. All the supernatant except for a small volume, not more than about 0.1 % of the total volume immediately above the cells was removed. The Hb concentration of the supernatant was determined.

The erythrocytes were then washed twice in 200 volumes of the isotonic buffer solution. The supernatants were analyzed for Hb. There was no further Hb loss in the experiments reported. In the cases where bottles were used small amounts of cells were observed floating on the surface. With test tubes which could be spun with greater centrifugal force no cells were detected in the supernatant. This was checked by phase contrast microscopy of the bottom portion of an aliquot of the

The following abbreviations are used: ATP, adenosine-triphosphate; NADH, reduced diphosphopyridine nucleotide; NADP, oxidized triphosphopyridine nucleotide; GSH, reduced glutathione; GSSG, oxidized glutahione; Cat., Catalase; G-6-PDH, glucose-6-phosphate dehydrogenase; HK, hexokinase; LDH, lactate dehydrogenase; Hb, hemoglobin.

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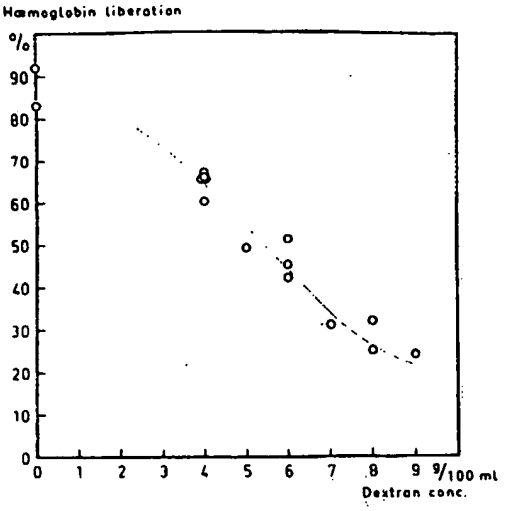

Fig. 1. Relation between hemoglobin liberation and dextran concentration in the hypotonic buffer solution.

supernatant after recentrifugation of the supernatant at 104,000 × g for 20 min. The loss of cells was small and very unlikely to have seriously affected the results since the average amount of Hb remaining in the cell fractions before the first washing and after the second was, within experimental error, the same.

After the last washing the hematocrit of the erythrocyte suspension was adjusted to about 50 per cent by making it up to a known volume with isotonic buffer. This final suspension was used for the determinations of enzyme activities, GSH-GSSG and ATP concentrations. The Hb concentration was determined and the cells were counted in a phase contrast microscope. In one experiment the activity of LDH was also determined in the first supernatant.

In all experiments a blood sample was run as a control by an identical procedure to that described above except that instead of the hypotonic solution an isotonic saline, pH 7.4 (NaCl 122 mM, KCl 5 mM, NaH_2PO_4 and Na_2HPO_4 20 mM) containing 6 per cent dextran was used.

Preparation of the samples for determination of enzyme activities, glutathione and adenosine-triphosphate concentrations

Aliquots of the final erythrocyte suspension were stored in liquid nitrogen, thawed at 0 °C and completely hemolyzed in hypotonic buffer. The hemolysate was used for the determination of enzyme activites. Another volume of the suspension was added to 10 volumes of ice cold 0.6 M perchloric acid for the ATP-determinations and to 10 volumes of ice cold 0.6 M perchloric acid with 2.5 per cent EDTA (w/v, disodium salt) for the determination of glutathione.

Cat.-activity was determined spectrophotometrically in a hydrogen peroxide system as according to Werner and Heider (1963), the activities of G-6-PDH, HK, and LDH in NADP- and NADH-dependent systems by the methods of Löhr and Waller (1962), Grignani and Löhr (1960) and Bergmeyer, Bernt and Hess (1963) respectively. GSH and GSSG were determined enzymatically with glyoxalase I and glutathione reductase as described by Klotzsch and Bergmeyer (1962) and modified for erythrocytes by Hjelm (1964). ATP was determined in a phosphoglycerate kinase — phosphoglyceraldehyde dehydrogenase system by the method of Adam (1963). The activities and concentrations were calculated for 10^{11} erythrocytes. The analyses were carried out with an Eppendorf photometer, connected via an Eppendorf scale expander to a recorder (Philips, model PR 2210 U) or a Beckman DB spectrophotometer with recorder (Sargent, model SR).

Results

1. *The relation between dextran concentration and hemoglobin liberation*

The relationship is shown in Fig. 1. There appears to be approximate linearity at least between 2 and 7 per cent dextran. The straight line in this region was calculated by the method of least squares.

2. *The relation between the dextran concentration and escape of different-sized molecules during partial hemoglobin liberation*

In Fig. 2 the loss of certain substances is shown in relation to the dextran concentration in the hypotonic solution. The Hb release shown in Fig. 1 is also redrawn in

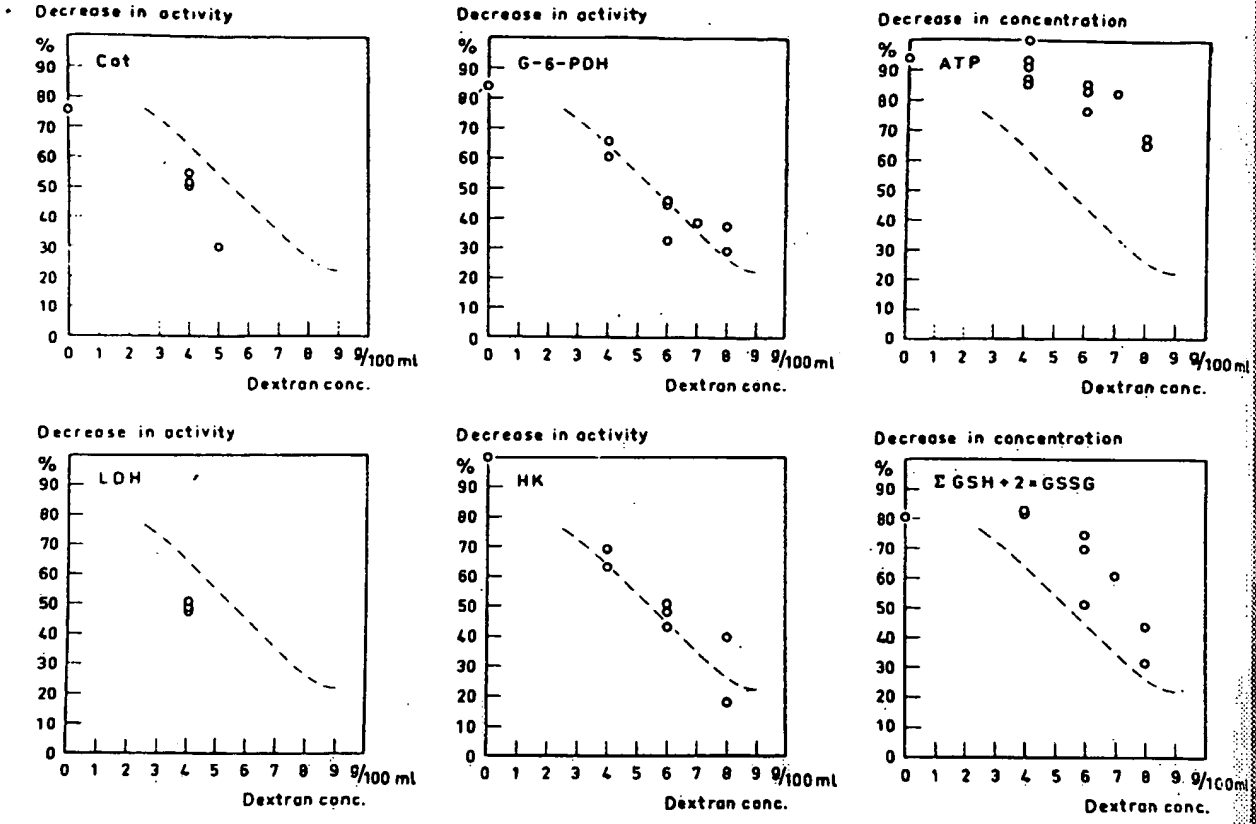

Fig. 2. The decrease of enzyme activities, glutathione and adenosinetriphosphate concentrations in relation to dextran concentration in the hypotonic buffer solution.
The hemoglobin release is shown as a line.

these graphs as a line. It is evident that there is a relation between the dextran concentration and the magnitude of disappearance of those components, measured at different dextran concentrations. There also appears to be a rough inverse correlation between the magnitude of loss and the molecular weight of a component. Thus the loss of ATP and GSH-GSSG (expressed as GSH-moities, i.e. GSH + 2 × GSSG) is greater than that of Hb while the enzyme losses were similar or less.

The activities of all substances except LDH (and Hb) were not measured in the supernatant because of experimental difficulties. It is however clear that LDH actually leaves the erythrocyte since the activity found in the supernatant corresponded rather well to that expected from its loss from the cell fraction. See Table I.

Discussion

There has been considerable discussion as to whether enzymes (and co-factors) are fixed to the cell stroma or are free to move out of the cell. In the experiments reported it can be concluded that all the substances studied were, at least partially, free to move out of the cell. Secondly there seems to be a tendency for molecular species with lower molecular weights or smaller molecular dimensions to escape to a greater extent. Hemolysis is however a complex process and the interpretation of "escape data" in terms of the molecular properties of the escapants is fraught with considerable difficulty.

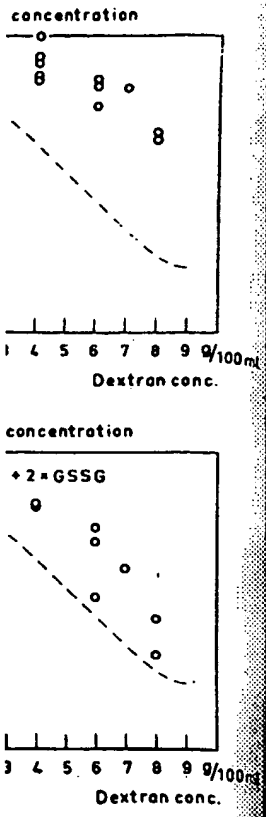

TABLE I. Recovery of lactate dehydrogenase in cell fraction and supernatant after partial hemoglobin liberation in one experiment

	Enzyme activity in international units				
Hemoglobin loss per cent	Activity per 10^{11} cells in final suspension	Activity in total volume of final suspension	Activity in total volume of first supernatant	Sum of activities in first supernatant and final suspension	Recovery of enzyme activity in first supernatant and final suspension combined per cent
0	236	10.5	0	10.5	100 (reference value)
0	255	10.5	0	10.5	100 (reference value)
66	126	5.4	3.3	8.7	83
66	131	5.6	1.1	6.7	64
66	136	5.8	1.8	7.6	72

The magnitude of the loss of a substance will depend on whether its movement is restricted spatially or temporally. It may be fixed to some structural part of the cell which will prevent some or all of it escaping. It may also be complexed to some other component with the result that the complex escapes at a different rate. Independently of any restriction imposed by a barrier such as a membrane, differences in diffusion rates will mean that the smaller the molecule the faster will it escape from the cell and as the hemolytic process is of a transient nature the limited time available may be expected to result in differences in relative final concentrations in the cell. Furthermore this effect will be exaggerated if the transient increase in porosity itself also exerts a molecular sieve effect (e.g. as with a heteroporous membrane).

If the increased porosity of the cell were of infinite duration allowing equilibration of all permeant species a cut off might be observed, indicating the maximum size of the "pore". Molecular species smaller than a critical "pore size" would thus have no restriction on their escape from the cell while larger molecules would not be able to escape. In the present instance, however, the porosity appears to be of too short a duration to allow equilibration and thus the pore size distribution may tend to impose its own graded sieving effect. For example if there is heteroporosity there is, other things being equal, a greater area available for the diffusion out of small than large molecules. It must be emphasized, however, that the results shown here do not necessarily indicate the presence of a membrane sieving effect.

Furthermore it is quite possible that bulk flow of water may occur during hemolysis. An outward bulk flow would tend to give similar values for the relative losses of different substances. An inward bulk flow, opposing an outward flux of solutes due to diffusion, might tend to restrain preferentially the escape of larger solutes.

The localization within the cell of the molecules studied is rather uncertain. The ghosts remaining after partial hemolysis in hypotonic dextran are almost certainly more or less intact cells. Teorell (1952), for example, showed that erythrocyte ghosts produced by mild hyposmotic trauma can function as osmometers, and Stein (1956)

TABLE II. Molecular weights of components studied

	Molecular weight	Reference
Cat	220,000 (human)	Herbert and Pinsent, 1948
G-6-PDH	74,000 (guinea pig)	Andrews, 1962
Hb	64,000 (human)	Braunitzer et al. 1961
HK	96,000 (yeast)	Kunitz and McDonald, 1946
LDH	110—120,000 (pig)	Wieland, Duesberg and Determan, 1963
ATP	507	
GSH	307	
GSSG	613	

found that the transport of glycerol and other non-electrolytes was similar in ghosts and unhemolysed cells. Many papers have also been published on the active transport of cations in ghosts showing their ability to accumulate and retain potassium (cf. Passow 1964). Bartlett (1958) pointed out that the usual way of obtaining "stroma" by osmotic hemolysis is not a very good way of studying enzyme localization. The interpretation of the results reported here will be much more difficult if we have to consider that some of the substances are fixed to immovable sites in the cell. It is generally regarded as difficult to remove all the Hb from a cell but Dodge, Mitchell and Hanahan (1963) showed that Hb can be removed completely under special conditions. If the red cell is considered as consisting of a thin bounding membrane containing an interior which is relatively structureless, the behaviour of Hb can then be regarded as representative for escaping species. The mass of Hb in the cell is so large that even if the inner surface of the membrane fixed a complete layer of Hb molecules this would constitute only to about 1—2 per cent of the Hb originally present in the cell and thus does not introduce a serious source of error.

In Table II values are given for molecular weights of the substances analysed. These values are compiled from the literature and must in some cases be regarded as rather approximate. The molecular weights values given for HK and Cat. are derived from ultracentrifugation data. In gel filtration through Sephadex® G-100 Cat. is slightly more excluded then LDH which has a molecular weight of 110,000—120,000 (Aebi *et al.* 1964). The values for LDH and G-6-PDH have been calculated from gel filtration data.

Cat. and LDH are larger than Hb and escape to a less extent than does Hb. HK and G-6-PDH have sizes similar to Hb and show a similar escape pattern. The tripeptide GSH has relatively small molecular dimensions (both in its reduced and oxidized forms) and leaves the cell to a greater extent than Hb. The same is true for ATP although the decrease in concentration could also be explained by an increased ATP-ase activity. This matter has not been seriously investigated but seems somewhat unlikely since there was no increase in adenosine-diphosphate and adenosine-monophosphate concentrations wich were measured in one case.

Taking all the components studied into account it appears that the relative amount

which escaped was inversely related to molecular size. In view of this relationship it is tempting to suggest that the greater part, at least, of each of these substances is not immovably fixed within the cell. Further, it appears that this method could be used for producing erythrocytes with reproducible differences in the intracellular composition. Such cells might be useful in studies of metabolic regulation mechanisms, especially of the glycolytic system.

The authors are indebted to Drs. N. V. B. Marsden and A. M. M. Zade-Oppen for valuable discussion and criticisms. Skilful technical assistance was given by Mrs. Birgitta Larsson, Miss Marja Mäkinen and Mr Staffan Håkansson. This study was supported by the Swedish Medical Research Council (Projects No. W 397 and Y 291), the Medical Faculty, University of Uppsala, Magnus Bergvalls Stiftelse, Svenska Sällskapet för Medicinsk Forskning.

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